

FEBS
Lettersjournal homepage: www.FEBSLetters.org

The antiviral protein viperin is a radical SAM enzyme

Kaitlin S. Duschene, Joan B. Broderick*

Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT 59717, USA
Astrobiology Biogeochemistry Research Center, Montana State University, Bozeman, MT 59717, USA

ARTICLE INFO

Article history:

Received 17 December 2009

Revised 8 February 2010

Accepted 16 February 2010

Available online 20 February 2010

Edited by Stuart Ferguson

Keywords:

Viperin

Antiviral

Radical SAM

Iron-sulfur cluster

Metalloenzyme

S-Adenosylmethionine

ABSTRACT

Viperin, an interferon-inducible antiviral protein, is shown to bind an iron-sulfur cluster, based on iron analysis as well as UV-Vis and electron paramagnetic resonance spectroscopic data. The reduced protein contains a $[4\text{Fe-4S}]^{1+}$ cluster whose g-values are altered upon addition of S-adenosylmethionine (SAM), consistent with SAM coordination to the cluster. Incubation of reduced viperin with SAM results in reductive cleavage of SAM to produce 5'-deoxyadenosine (5'-dAdo), a reaction characteristic of the radical SAM superfamily. The 5'-dAdo cleavage product was identified by a combination of HPLC and mass spectrometry analysis.

© 2010 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.

1. Introduction

Viperin (virus inhibitory protein, endoplasmic reticulum-associated, interferon-inducible) has been shown to be strongly induced by both type I and II interferons in response to a variety of different viral infections [1–7], however the specific means by which viperin acts to carry out its antiviral function has yet to be identified. Insight into a possible mode of action for viperin was provided by the recent demonstration of its interaction with farnesyl diphosphate synthase (FPPS), an enzyme that is essential for isoprenoid biosynthesis, including squalenes and sterols [7,8]. Wang et al. provided evidence that the intracellular interaction of viperin with FPPS decreases the activity of FPPS, ultimately disrupting the formation of lipid rafts and thereby increasing the lateral mobility of the plasma membrane [7]. However, the specific biochemical interactions between viperin and FPPS, and the roles these might play in the pathways downstream of FPPS leading up to isoprenoid biosynthesis, remain to be identified.

The finding of a relationship between viperin function and lipid raft disruption was significant due to the important roles lipid rafts play in the life cycles of a number of viruses, including both HIV

and the influenza virus [9]; thus lipid raft disruption could be a novel mode of antiviral activity [7]. A simplified illustration of an immune system response to viral infection is shown in Fig. 1, wherein viral infection triggers upregulation of several genes leading to production of interferon regulatory factor 3 (IRF3), which stimulates interferon (IFN) production. Secreted IFNs bind to type I IFN receptors (IFNR) and activate formation of IFN stimulated gene factor 3 (ISGF3), which then triggers the IFN stimulated response element (ISRE) of the gene promoter. In turn, genes for numerous antiviral proteins, including viperin, are upregulated. Viperin (shown as red boxes) migrates to the cytosolic face of the endoplasmic reticulum (ER) where it can interact with FPPS (shown as green circles) [7]. Viperin binding with FPPS leads to disruption of lipid raft domains thereby forming viral buds with “stalk-like” or “daisy chain” structures, which cannot be released from the plasma membrane [7,10].

Viperin has been suggested to be a member of the radical S-adenosylmethionine (SAM) superfamily due to the presence of a $\text{CX}_3\text{CX}_2\text{C}$ motif, as well as some additional motifs found in other radical SAM enzymes [11]. However, the biochemical characteristics and the precise function of viperin remain unknown. Sequence analysis suggests that human viperin is a three-domain protein, with the radical SAM domain (residues 77–209) flanked by a leucine zipper domain (residues 1–76), and a C-terminal domain [12]. The leucine zipper domain may be responsible for proper folding of the protein as well as anchoring the protein to the cytoplasmic side of the ER [13].

Abbreviations: SAM, S-adenosylmethionine; EPR, electron paramagnetic resonance; 5'-dAdo, 5'-deoxyadenosine; SAH, S-(5'-adenosyl)-L-homocysteine; MTA, methylthioadenosine; DAF, 5-deazariboflavin

* Corresponding author. Fax: +1 406 994 5407.

E-mail address: jbroderick@chemistry.montana.edu (J.B. Broderick).

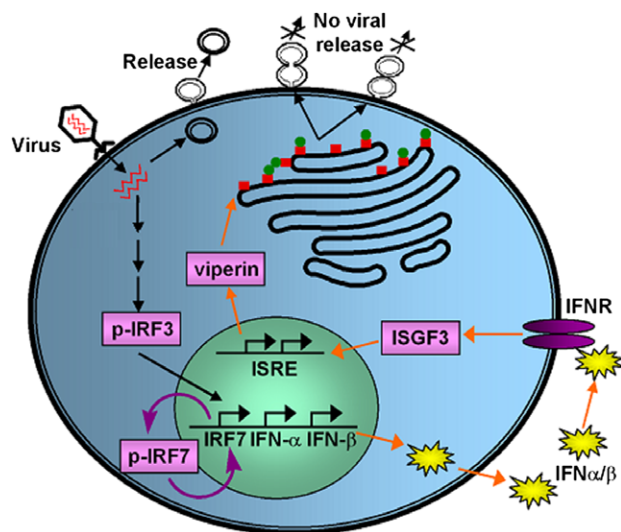


Fig. 1. Schematic illustration of a representative immune response pathway that leads to the disruption of viral release from the plasma membrane.

We report here the first spectroscopic and biochemical evidence that human viperin is a radical SAM enzyme that contains the characteristic [4Fe-4S] cluster and catalyzes the reductive cleavage of SAM. The identification of viperin as a radical SAM enzyme provides a foundation for understanding its specific mode of action in the immune response to viral infection.

2. Materials and methods

2.1. Materials

S-Adenosylmethionine was synthesized from methionine and ATP using SAM synthetase and subsequently purified using cation exchange chromatography as previously described [14]. All other chemicals were obtained from commercial sources.

2.2. Cloning of viperin

The cDNA for human viperin (*cig5*) lacking the N-terminal sequence encoding the leucine zipper (cDNA provided in pcDNA3.1) was received as a generous gift from Peter Cresswell at Yale University; the *cig5* cDNA in pLNCX2 was also received as a generous gift from Michael Beard at the University of Adelaide, Australia. *Cig5* was PCR amplified from pcDNA3.1. The PCR fragments were purified, digested and ligated into a pET-14b vector (Novagen) containing an N-terminal 6x-Histidine affinity tag (His₆) (see [Supplementary material](#)).

2.3. Viperin expression, purification, and reconstitution

Viperin lacking the leucine zipper domain (viperin_{43–340} hereafter) was overproduced in *Escherichia coli* Rosetta(DE3)pLysS cells (Novagen). Growth of cell lines and protein expression was performed as described in [Supplementary material](#). Lysis, purification and reconstitution procedures were all carried out under anaerobic conditions in an anaerobic chamber (Coy Laboratories) (see [Supplementary material](#)). Protein concentrations were determined by the Bradford method using bovine serum albumin as the standard [15]. Iron content was evaluated spectrophotometrically [16].

2.4. Spectroscopic characterization

For UV–Vis absorption experiments, samples were transferred to an anaerobic cuvette within an anaerobic chamber (Mbraun)

containing 1 ppm or less of O₂. Room temperature UV–Vis absorption data were acquired using a Cary 6000i UV–Vis/near-IR spectrophotometer (Varian) with Spectrosil 1.4 ml anaerobic cuvettes (Starna). Low temperature X-band CW (continuous wave) electron paramagnetic resonance (EPR) spectra were recorded using a Varian E-109 spectrometer modified with a National Instruments computer interface (for data collection and field control) and equipped with an Air Products and Chemicals LTD-3-110 Heli-Tran Liquid Helium Transfer Refrigerator (Allentown, PA). EPR parameters were as follows: sample temperature 12.5 K; microwave frequency 9.24 GHz; microwave power 2 mW; time constant 0.50; each spectrum shown is the average of three scans.

2.5. Reduction of viperin

Reduction of viperin was carried out in an anaerobic chamber (Mbraun). Reaction mixtures consisted of 5 mM DTT, 50–100 mM Tris pH 7.0, 58–135 μM of as-isolated or reconstituted viperin in HEPES buffer pH 7.0, and 50–100 μM 5-deazariboflavin (DAF) or 1 mM dithionite depending on method of reduction. Mixtures were prepared by combining reagents from anoxic stock solutions in the order listed above, with DAF or dithionite always added last. Reactions using DAF were illuminated with a 300 W halogen lamp for 1 h and kept cool during illumination by immersion in an ice water bath. Reductions using dithionite were incubated on ice for 10 min. After the reduction of viperin, SAM (3 equiv to protein) was added to the samples and allowed to incubate for 5–10 min. Samples to be used for EPR analysis were placed in an EPR tube and flash frozen. Samples to be used for UV–Vis analysis were immediately transferred to an anaerobic cuvette and analyzed spectroscopically.

2.6. In vitro SAM cleavage assay

SAM cleavage samples were prepared anaerobically. The detection of the cleavage and degradation products including 5'-deoxyadenosine, S-(5'-adenosyl)-L-homocysteine and methylthioadenosine was accomplished using HPLC and confirmed by mass spectrometry (see [Supplementary material](#)).

3. Results and discussion

3.1. Purification and biochemical properties of viperin

The cloning of *cig5* (minus the N-terminal region encoding the putative membrane anchor) into the pET14b vector was confirmed with restriction enzyme digestion. Synthesizing viperin_{43–340} from the bacterial plasmid did not result in significant overproduction (Fig. S1), even though the *E. coli* Rosetta(DE3)-pLysS cells, containing some of the rare codons found in mammalian genes but absent in bacteria, were used. Large scale growths generally yielded ~25 mg of protein per 60 g of cell paste. The instability of the viperin_{43–340} protein led to high levels of precipitation when concentrated to levels >250 μM or during repeated freeze-thaw cycles. The observed instability of viperin_{43–340} could be related to the removal of the N-terminal domain during the cloning process. Hinson and Cresswell showed that the lack of the N-terminal domain could negatively affect protein dimerization [13], and it is clear that the lack of this domain prevents membrane localization; either or both of these effects could have an impact on overall protein stability. In addition, viperin contains several putative N-glycosylation sites in the radical SAM domain, and these would not be glycosylated upon expression in a bacterial host; the lack of glycosylation could also have an effect on protein stability.

3.2. Viperin binds a reducible [4Fe-4S] cluster

Purified viperin_{43–340} was found to contain iron (0.9 ± 0.2 mol Fe/mol viperin) and was dark yellow to light brown in color at concentrations of ~ 100 μ M. After reconstitution, the protein became dark brown in color at similar concentrations and contained an enhanced amount of iron (3.7 ± 0.1 mol Fe/mol viperin). The UV–Vis absorption spectra for the purified as-isolated and reconstituted viperin_{43–340} samples show broad overlapping ligand-to-metal charge transfer (LMCT) transitions characteristic of iron-sulfur clusters, including a sharp feature at ~ 315 nm and a broad peak between ~ 370 and 450 nm, both of which are typical features for proteins containing [4Fe-4S]²⁺ clusters (Fig. 2A) [17–19]. The reconstituted enzyme exhibits higher intensity for the LMCT features, consistent with its higher iron numbers, as well as an additional feature at ~ 615 nm, perhaps indicative of the presence of [2Fe-2S]²⁺ clusters (Fig. 2A) [20]. Reduction of the reconstituted protein with either dithionite or photoreduced 5-deazariboflavin results in decreased intensity throughout the visible region (Fig. 2B), which is consistent with the reduction of a [4Fe-4S]²⁺ cluster to a [4Fe-4S]¹⁺ cluster. Addition of SAM to the reduced enzyme results in a slight decrease in intensity, but no other significant changes in the visible absorption spectrum (Fig. 2B).

Reconstituted viperin_{43–340} exhibits a nearly isotropic electron paramagnetic resonance (EPR) signal characteristic of a [3Fe-4S]¹⁺ cluster and accounting for 0.09 spins/protein (Fig. 3A); the fact that this signal accounts for only $\sim 10\%$ of the iron in the sample indicates that the remainder is in an EPR silent form, most likely primarily [4Fe-4S]²⁺ clusters. Such [3Fe-4S]¹⁺ clusters are frequently observed for radical SAM enzymes, and are believed to result from oxidative loss of the “unique” iron of the [4Fe-4S] cluster [21–24]. Photoreduction of reconstituted viperin_{43–340} results in loss of the [3Fe-4S]¹⁺ signal and appearance of a nearly axial EPR signal with g -values ($g = 2.02, 1.92, 1.91$; 0.28 spins/protein) characteristic of [4Fe-4S]¹⁺ clusters (Fig. 3B); this signal is observed only below ~ 40 K, as would be expected for a [4Fe-4S]¹⁺ cluster. Addition of SAM alters the EPR signal ($g = 2.03, 1.95$ and 1.88 ; 0.28 spins/protein Fig. 3C), consistent with the coordination of SAM to the [4Fe-4S] cluster as observed for other radical SAM enzymes [25,26]. Together, the UV–Vis and EPR spectroscopic data provide the first clear evidence that viperin binds an iron-sulfur cluster with properties characteristic of the radical SAM enzymes.

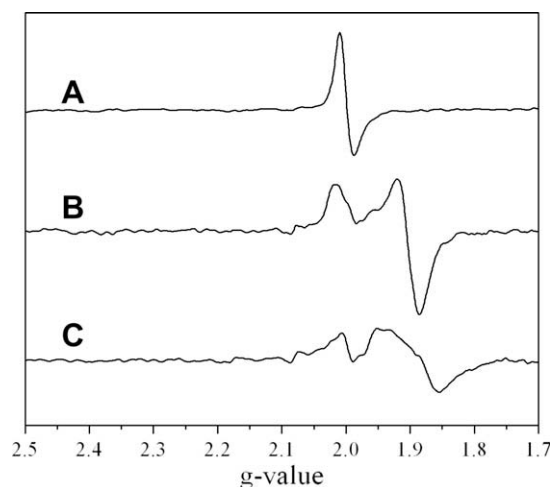


Fig. 3. X-band EPR spectra of reconstituted viperin (96 μ M). (A) As reconstituted. (B) Reconstituted and photoreduced. (C) Reconstituted and photoreduced followed by addition of SAM.

3.3. Viperin catalyzes the reductive cleavage of SAM

The most characteristic feature of the radical SAM superfamily enzymes is their ability to catalyze the reductive cleavage of SAM to yield methionine and a 5'-deoxyadenosyl radical intermediate (5'-dAdo[•]). In the normal catalytic reactions, the 5'-dAdo[•] abstracts a hydrogen atom from substrate to initiate subsequent transformations. Many of these enzymes, however, exhibit uncoupled SAM cleavage, whereby even in the absence of substrate the production of methionine and 5'-deoxyadenosine (5'-dAdo) can be observed under reducing conditions. Because the substrate for viperin is currently unknown, we examined the ability of viperin_{43–340} to catalyze uncoupled reductive cleavage of SAM.

Fig. 4A shows that in the absence of viperin, SAM remains uncleaved with one major peak eluting at 2.4 min (SAM) along with a minor peak (11.0 min) representing a small amount of degradation of SAM into methylthioadenosine (MTA). In the presence of viperin_{43–340}, HPLC analysis demonstrates the appearance of two new peaks eluting at 5.8 and 6.5 min, which correspond to the elution times of S-(5'-adenosyl)-L-homocysteine (SAH) and 5'-dAdo,

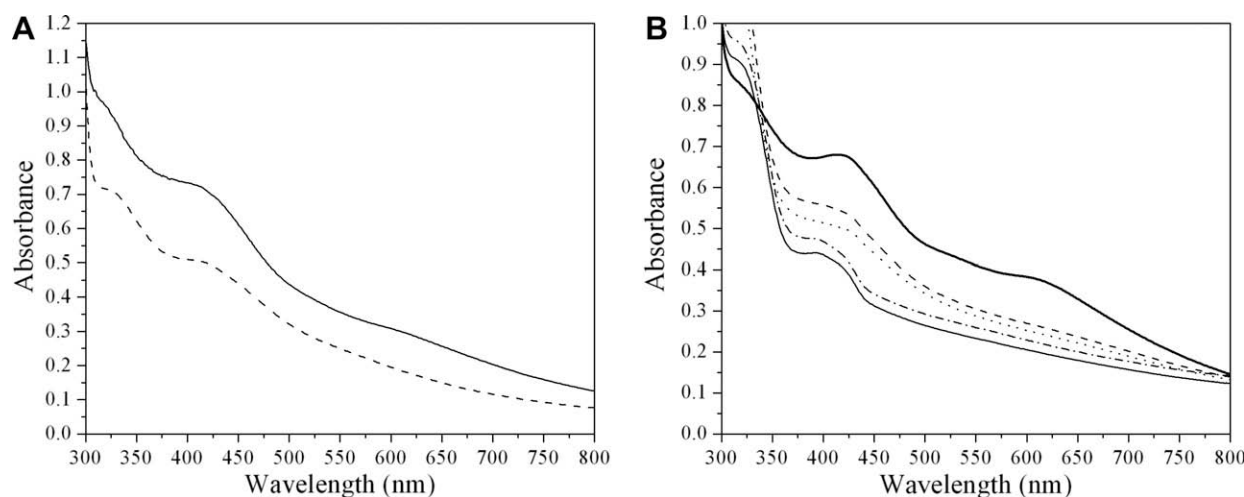


Fig. 2. UV–Vis spectroscopic characterization of viperin. (A) As-isolated (dashed line, normalized to 100 μ M, 0.7 Fe/protein) or reconstituted (solid line, 100 μ M, 2.5 Fe/protein). (B) Reconstituted (normalized to 42 μ M, 3.7 Fe/protein, thick solid line), reduced with dithionite (dashed line), reduced by photoillumination (dashed-dotted line), reduced by photoillumination followed by the addition of SAM (dotted line), reduced by photoillumination followed by the addition of SAM (thin solid line).

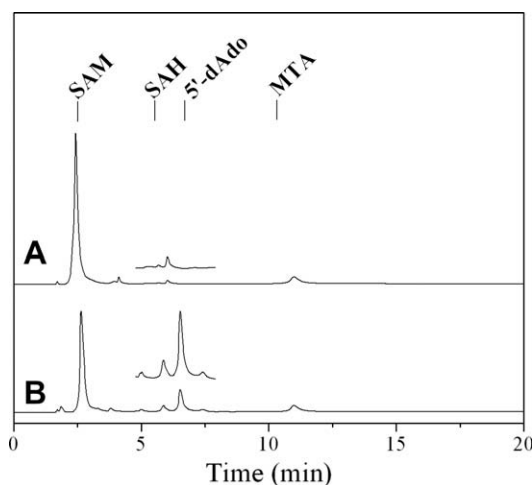


Fig. 4. HPLC analysis of SAM cleavage assay. (A) Control assay without viperin. The elution times of standard solutions, as shown above by the labeled tick marks, are: S-adenosylmethionine (SAM) at 2.5 min, S-(5'-adenosyl)-L-homocysteine (SAH) at 5.5 min, 5'-deoxyadenosine (5'-dAdo) at 6.7 min, and methylthioadenosine (MTA) at 10.3 min. (B) Assay containing viperin. Inset: expanded region between 5 and 8 min highlighting the appearance of 5'-dAdo.

respectively (Fig. 4B). To confirm the identity of the compounds eluting at 5.8 and 6.5 min, the peaks were collected and subjected to electrospray-ionization (ESI) mass spectrometry. MS analysis for the sample collected at 5.8 min provided evidence for the presence of both SAH [major component, $m/z = 384.9$ (M+H)] and 5'-dAdo [minor component, $m/z = 249.0$ (M-2H), $m/z = 252.1$ (M+H)]. The peak eluting at 6.5 min gave similar results by MS analysis with a peak corresponding to 5'-dAdo [major component, $m/z = 249.0$ (M-2H), $m/z = 252.2$ (M+H)] as well as a peak for SAH [minor component, $m/z = 385.0$ (M+H)].

3.4. Conclusion

We demonstrate here that viperin_{43–340} binds a reducible [4Fe-4S] cluster and catalyzes the reductive cleavage of SAM, thus pointing directly to a role for radical SAM chemistry in the antiviral response. Shaveta et al. recently reported that viperin is a radical SAM enzyme, however the only evidence presented was a brown color and the corresponding UV-Vis spectrum after reconstituting viperin [27]. Unlike our work reported herein, Shaveta et al. did not remove excess iron and sulfide after reconstitution; as a result, the UV-Vis data indicates a huge excess of adventitious iron-sulfur clusters, as the extinction coefficient is an order of magnitude higher than typically observed for a single [4Fe-4S] cluster per protein [27]. The iron content of the reconstituted protein examined by Shaveta et al. was not reported, and thus quantitative comparisons to our results cannot be made. Regardless, the observation of binding of iron and sulfide to a protein does not constitute evidence that the protein belongs to the radical SAM superfamily. Our current results demonstrating the binding of a reducible [4Fe-4S] cluster that interacts with SAM, as evidenced by spectroscopy, and that reductively cleaves SAM, as evidenced by HPLC/MS, does provide clear evidence that viperin is a radical SAM enzyme.

The radical SAM superfamily enzymes catalyze a diverse range of reactions, all of which are initiated by a hydrogen atom abstraction from substrate [28]. Given the evidence that viperin inhibits FPPS and disrupts lipid raft formation, two particular classes of radical SAM reactions seem particularly relevant in providing insights into the mode of action of viperin. First, the glyceryl radical

activating enzymes provide precedent for radical SAM enzyme-catalyzed protein modification [28], which could be a basis for inactivation of FPPS by viperin. Second, radical SAM enzymes catalyzing alkane functionalization [29] support the possibility of viperin-catalyzed functionalization of a metabolite downstream of FPPS; such modification could disrupt cholesterol biosynthesis or the ability of the functionalized cholesterol to form lipid rafts. While the substrate for viperin has yet to be identified, the results presented herein provide significant new insight into the chemistry underlying the antiviral response mediated by viperin.

Acknowledgements

The authors gratefully acknowledge Peter Cresswell for providing us with *cig5* cDNA. This research has been supported by the NIH (GM54608 to J.B.B. and RR-020185), and by the Montana NSF EPSCOR (EPS-0701906). The Astrobiology Biogeocatalysis Research Center is supported by the NASA Astrobiology Institute (NAI05-19).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.02.041.

References

- [1] Boudinot, P., Riffault, S., Salhi, S., Carrat, C., Sedlik, C., Mahmoudi, N., Charley, B. and Benmansour, A. (2000) Vesicular stomatitis virus and pseudorabies virus induce a *vig5/cig5* homologue in mouse dendritic cells via a different pathway. *J. Gen. Virol.* 81, 2675–2682.
- [2] Chin, K.-C. and Cresswell, P. (2001) Viperin (*cig5*), an IFN-inducible antiviral protein directly induced by human cytomegalovirus. *Proc. Natl. Acad. Sci. USA* 98, 15125–15130.
- [3] Helbig, K., Lau, D.-Y., Semendric, L., Harley, H. and Beard, M. (2005) Analysis of ISG expression in chronic hepatitis C identifies viperin as a potential antiviral effector. *Hepatology* 42, 702–710.
- [4] Khaiboullina, S., Rizvanov, A., Holbrook, M. and St Jeor, S. (2005) Yellow fever virus strains Asibi and 17D-204 infect human umbilical cord endothelial cells and induce novel changes in gene expression. *Virology* 342, 167–176.
- [5] Rivieccio, M., Suh, H.-S., Zhao, Y., Zhao, M.-L., Chin, K.-C., Lee, S. and Brosnan, C. (2006) TLR3 ligation activates an antiviral response in human fetal astrocytes: a role for viperin/*cig5*. *J. Immunol.* 177, 4735–4741.
- [6] Severa, M., Coccia, E. and Fitzgerald, K. (2006) Toll-like receptor-dependent and -independent viperin gene expression and counter-regulation by PRD1-binding factor-1/BLIMP1. *J. Biol. Chem.* 281, 26188–26195.
- [7] Wang, X., Hinson, E. and Cresswell, P. (2007) The interferon-inducible protein viperin inhibits influenza virus release by perturbing lipid rafts. *Cell Host Microbe* 2, 96–105.
- [8] Ashby, M. and Edwards, P. (1989) Identification and regulation of a rat liver cDNA encoding farnesyl pyrophosphate synthetase. *J. Biol. Chem.* 264, 635–640.
- [9] Ono, A. and Freed, E.O. (2005) Role of lipid rafts in virus replication in: *Advances in virus research* (Roy, P., Ed.), pp. 311–358, Elsevier Academic Press, San Diego.
- [10] Waheed, A.A. and Freed, E.O. (2007) Influenza virus not cRAFTy enough to dodge viperin. *Cell Host Microbe* 2, 71–72.
- [11] Sofia, H.J., Chen, G., Hetzler, B.G., Reyes-Spindola, J.F. and Miller, N.E. (2001) Radical SAM, a novel protein superfamily linking unresolved steps in familiar biosynthetic pathways with radical mechanisms: functional characterization using new analysis and information visualization methods. *Nucleic Acids Res.* 29, 1097–1106.
- [12] Jiang, D., Guo, H., Xu, C., Chang, J., Gu, B., Wang, L., Block, T. and Guo, J.-T. (2008) Identification of three interferon-inducible cellular enzymes that inhibit the replication of hepatitis C virus. *J. Virol.* 82, 1665–1678.
- [13] Hinson, E. and Cresswell, P. (2009) The N-terminal amphipathic α -helix of viperin mediates localization to the cytosolic face of the endoplasmic reticulum and inhibits protein secretion. *J. Biol. Chem.* 284, 4705–4712.
- [14] Walsby, C., Hong, W., Broderick, W., Cheek, J., Ortillo, D., Broderick, J. and Hoffman, B. (2002) Electron-nuclear double resonance spectroscopic evidence that S-adenosylmethionine binds in contact with the catalytically active [4Fe-4S]⁺ cluster of pyruvate formate-lyase activating enzyme. *J. Am. Chem. Soc.* 124, 3143–3151.
- [15] Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
- [16] Fish, W. (1988) Rapid colorimetric micromethod for the quantitation of complexed iron in biological samples. *Meth. Enzymol.* 158, 357–364.

- [17] Hagen, K., Watson, A. and Holm, R. (1983) Synthetic routes to iron sulfide (Fe_2S_2 , Fe_3S_4 , Fe_4S_4 , and Fe_6S_9), clusters from the common precursor tetrakis(ethanethiolate)ferrate(2-) ion ($[\text{Fe}(\text{SC}_2\text{H}_5)_4]^{2-}$): structures and properties of $[\text{Fe}_3\text{S}_4(\text{SR})_4]^{3-}$ and bis(ethanethiolate)nonathiohexaferrate(4-) ion ($[\text{Fe}_6\text{S}_9(\text{SC}_2\text{H}_5)_2]^{4-}$), examples of the newest types of Fe-S-SR clusters. *J. Am. Chem. Soc.* 105, 3905–3913.
- [18] Kulzer, R., Pils, T., Kappl, R., Hutterman, J. and Knappe, J. (1998) Reconstitution and characterization of the polynuclear iron-sulfur cluster in pyruvate formate-lyase-activating enzyme. *J. Biol. Chem.* 273, 4897–4903.
- [19] Ugulava, N., Frederick, K. and Jarrett, J. (2003) Control of adenosylmethionine-dependent radical generation in biotin synthase: a kinetic and thermodynamic analysis of substrate binding to active and inactive forms of BioB. *Biochemistry* 42, 2708–2719.
- [20] Ugulava, N., Gibney, B. and Jarrett, J. (2001) Biotin synthase contains two distinct iron-sulfur cluster binding sites: chemical and spectroelectrochemical analysis of iron-sulfur cluster interconversions. *Biochemistry* 40, 8343–8351.
- [21] Broderick, J. et al. (2000) Pyruvate formate-lyase activating enzyme: strictly anaerobic isolation yields active enzyme containing a $[3\text{Fe-4S}]^+$ cluster. *Biochem. Biophys. Res. Commun.* 269, 451–456.
- [22] Petrovich, R., Ruzicka, F., Reed, G. and Frey, P. (1992) Characterization of iron-sulfur clusters in lysine 2,3-aminomutase by electron paramagnetic resonance spectroscopy. *Biochemistry* 31, 10774–10781.
- [23] Krebs, C., Broderick, W.E., Henshaw, T.F., Broderick, J.B. and Huynh, B.H. (2002) Coordination of adenosylmethionine to a unique iron site of the $[4\text{Fe-4S}]$ of pyruvate formate-lyase activating enzyme: a Mössbauer spectroscopic study. *J. Am. Chem. Soc.* 124, 912–913.
- [24] Krebs, C., Henshaw, T.F., Cheek, J., Huynh, B.-H. and Broderick, J.B. (2000) Conversion of 3Fe-4S to 4Fe-4S clusters in native pyruvate formate lyase activating enzyme: Mössbauer characterization and implications for mechanism. *J. Am. Chem. Soc.* 122, 12497–12506.
- [25] Liu, A. and Gräslund, A. (2000) Electron paramagnetic resonance evidence for a novel interconversion of $[3\text{Fe-4S}]^+$ and $[4\text{Fe-4S}]^+$ clusters with endogenous iron and sulfide in anaerobic ribonucleotide reductase activase in vitro. *J. Biol. Chem.* 275, 12367–12373.
- [26] Walsby, C., Ortillo, D., Yang, J., Nnyepi, M., Broderick, W.E., Hoffman, B.M. and Broderick, J.B. (2005) Spectroscopic approaches to elucidating novel iron-sulfur chemistry in the “Radical SAM” protein superfamily. *Inorg. Chem.* 44, 727–741.
- [27] Shaveta, G., Shi, J., Chow, V.T.K. and Song, J. (2010) Structural characterization reveals that viperin is a radical S-adenosyl-L-methionine (SAM) enzyme. *Biochem. Biophys. Res. Commun.* 391, 1390–1395.
- [28] Frey, P., Hegeman, A. and Ruzicka, F. (2008) The radical SAM superfamily. *Crit. Rev. Biochem. Mol. Biol.* 43, 63–88.
- [29] Booker, S.J. (2009) Anaerobic functionalization of unactivated C–H bonds. *Curr. Opin. Chem. Biol.* 13, 58–73.